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Original Article

Effects of orchids (*Orchis anatolica*) on reproductive function and fertility in adult male mice

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Aims: The aim of the present study was to determine the effect of orchid bulbs on the reproductive system of male albino mice.

Methods: Orchid bulb extract was fed to 20 male albino mice (5 g/mouse/day) for 35 days and compared with a similar number of mice as controls. Total testicular germ cell population, histometrical parameters, serum blood biochemistry and hormonal assay were determined.

Results: The ingestion of orchid bulb by mice induced a significant increase in the following parameters: (i) testes and seminal vesicle weights; (ii) number of different testicular germ cell population including interstitial Leydig cells and fibroblasts; and (iii) testicular cell linage dynamics obtained from testes and cauda epidydimides. An important finding was that the ingestion of the orchid diet by male mice increased their fertility. This was indicated by an elevation in the number of impregnated females when allowed to mate with treated mice, an increase in the impregnation sites, and an increase in the number of viable fetuses and the offspring's male/female ratio. A slight significant increase in the testosterone and follicular stimulating hormone titers in the treated mice were found in their blood serum. In contrast, a decrease in the number of degenerating cells was observed.

Conclusions: Orchid bulb treatment might play an important role in improving male reproductive potential and fertility. (Reprod Med Biol 2006; 5: 269–276)

Key words: adult mice, fertility, orchids (*Orchis anatolica*), reproductive system, spermatogenesis.

INTRODUCTION

HERBAL MEDICINE HAS long been used as a tool to produce natural drugs. In recent years the use of traditional medicine, enriched by information from plant research, has gained considerable interest.[1] The use of orchids in herbal medicine has a very long history and it is one of the most widespread of all plant families.[2,3] Orchids are found in many countries such as Greece, Turkey and Jordan. The orchid is a cool terrestrial plant that is usually grown in homes and gardens for its beauty, exoticism and the fragrance of its flowers. Although cultivated since the times of Confucius (c.551–479 BC), their commercialization in Europe started only at the end of the 18th century. The orchid blooms in the spring and it is a 5–10 cm long plant, with loose roots resembling small potatoes (tuberous roots). The Anatolica orchid, named scientifically, *Anatolisches knabenkuraut*, is a rather delicate-looking species with a loose spike of pinkish flowers with large lips and long prominent spurs, growing mostly in light-shaded pine forests.[4]

Several orchid species were listed in early Chinese medicine.[5] A good correlation was observed between the temperature characteristic of *Qi* and the ability of the Chinese herbs to produce or scavenge superoxides produced by tissues.[6,7] These observations led many researchers to use different types of orchids previously used in Chinese medicine, such as *Dendrobium nobile* and *Bletilla striata*, for different research purposes. Both species used were considered to be mildly 'cold' and to produce a considerable amount of superoxides, and the latter also has antimicrobial properties.[7] Since the Middle Ages, orchids have been popular for their supposed aphrodisiac properties.[8] Special concoctions of the tuberous roots and fleshy leaves of some species were considered to be sexual stimulants and even to help

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to produce male babies. A number of plant species have been tested for fertility regulation over the past 50 years and have subsequently been fortified by national and international agencies.[8,9] That is how they became a synonym of fertility and virility.[3,10–13] Previous studies based on earlier reports by Kerr and Lopez,[14] and Van der Pijl and Dodson[2] indicated that some orchid genera were considered to be pseudocopulatory. More recently, the pseudocopulatory effect has also been reported for *Tolumnia henekenii* (*Oncidiinae*, referred to as *Oncidium henekenii*).[15]

In the light of these facts, the objectives of the present study were to show and monitor the effects of the *Orchis anatolica* genera of the *Orchidaceae* family on reproductive organ weight, testicular germ cells population, and sperm motility and fertility. The present study might provide additional evidence of the effect of this plant on male fertility, thus suggesting that ingredients from this plant might be used to create a safe drug that increases male sexual potential.

MATERIALS AND METHODS

Animals and experimental design

A TOTAL OF 40 adult male and 40 adult female Swiss strain mice (*Mus musculus*) weighing 40 g were used in the present study. The mice were raised under the same conditions at the animal house unit at Jordan University of Science and Technology. Mice were kept under controlled temperature of 21 ±°C and 12 h light/12 h darkness schedule until the beginning of the study. Food and water were available ad libitum.

Treatment (extract preparation)

Large sized roots of *Orchis anatolica* were obtained from a local market and used in the present study. The orchid roots were dried at 33°C for 24 h using an electrical oven and an extract was made. This extract was given orally to male mice in a dose of 5 g/mouse/day in one single morning dose for 35 days.

Mice were divided into two groups and caged separately. Group 1 (control group): 20 male mice were used in this group as control. Mice received regular food without orchid roots for the length of their reproductive cycle (35 days). After this period, 10 mice were randomly chosen from this group and were allowed to mate with 20 control vehicle female mice, each male mouse was caged with two female mice separately; and Group 2 (experimental group): 20 male mice were used in this group and were treated with a dose of 5 g/mouse/day extract of dried large sized orchid roots for 35 days. After that, 10 male mice were allowed to mate with 20 untreated female mice and each male mouse was caged with two female mice separately.

Parameters studied

Body and organs weights

Bodyweights from both treated and control groups were recorded by the beginning and at the end of the experimental periods. After 35 days, at the end of their reproductive cycle, the mice were killed and the reproductive organs were dissected out, trimmed free of fat and each organ (testes, epididymides, seminal vesicle and vas deferens) was weighed individually on an electronic balance scale and immersed in 10% buffered formalin for further histological analysis.

Sperm count

To determine the sperm count, a small amount of cauda epididymal suspension was sucked onto the WBC pipette (up to 0.5 pipette mark). A 5% sodium bicarbonate ($NaHCO_3$) solution was added to this suspension until mark 11 was reached. The solution was shacked well and a drop of this new suspension was placed gently on a clean Neubauer chamber and covered with a clean cover slip. Using a light microscope at 100 magnification power, sperms were counted in 64 small WBC squares and the total number of sperms was calculated as millions/mL using this formula:

$$\text{Number of sperms (millions/mL)} = \frac{N \times 20 \times 1000}{0.4}$$

Dilution of 0.5 into 11 mark = a dilution of 20 times

Sperm motility

To determine the sperm motility, a 10 mg piece of Cauda epididymis was taken before dissection, cleaned and placed in a 0.2 mL physiological saline. Cauda epididymis was teased gently to release the sperm content in this saline. A drop of this suspension containing sperm was placed on the Neubauer chamber for observation and counting. Using a light microscope at 100 magnification power, motile spermatozoa along with the number of immotile spermatozoa were recorded within four squares constituting one field of the chamber (A, B, C and D). A total number of 20 separate fields were scored and the percentage of sperm with normal motility was calculated using this formula:

$$\text{Percentage of motile sperm} = \frac{\text{Number of motile sperms}}{\text{Total number of spermatozoa}} \times 100$$

Motility and sperm count obtained from both control and treated groups of mice were calculated using Prasad *et al.*[16] method and expressed as percentage motility and counted as millions/mL, respectively.

Fertility test

The fertility test was carried out in the cohabiting estrous or pro-estrous phase using the female mice that were left in the same cage with the treated male mice in a ratio of two females to one male after 35 days according to the WHO protocol.[17] The next morning after caging with male mice, a vaginal smear was taken to test for the presence of sperm so as to ensure that mating had occurred and this was considered to be day 1 of pregnancy. On day 16 of pregnancy and after pregnancy tests were confirmed, 10 female rats were killed and autopsied. The implantation and absorptions sites within the dissected uterus obtained from each female mouse were counted and recorded. The other 10 pregnant female rats were allowed to complete their pregnancy period to determine the male/female offspring's ratio.

Testicular cell population observation

Spermatogenic elements including spermatogonia, spermatocytes and spermatids were counted using 5 μm thick histological cross-sections of seminiferous tubules obtained from 10 male mice testis from both groups. All raw counts were transformed to 'true' counts by an adaptation of the Abercrombie formula[18] from germ cell diameter measurement. Interstitial cell types (such as fibroblast, immature and mature Leydig cells and degenerating cells) were estimated, applying a differential count over a population of 200 cells, and were statistically verified by the binomial distribution.[19]

Evaluation of male mouse fertility

To evaluate male mouse fertility, 40 female mice were used and divided into two groups. The first group contained 20 female mice that had been left to be fertilized by 10 male treated mice. The second group contained the same number of female mice that had been left to be fertilized by 10 male untreated control mice. In both groups, two female mice were caged together with only one male mouse and left to mate. After 16 days of pregnancy, 10 female mice from each group was randomly chosen and killed before completing their pregnancy period. The uterus of each female mouse was dissected out and the implantation sites as well as the viable fetuses within each uterus were counted and recorded. The remaining 10 female mice from both groups were left to complete their pregnancy period and then the offspring ratio of male to female mice was evaluated in both groups.

Serum biochemistry and hormonal assays

Blood was obtained from mice by direct cardiac puncture after anesthesia. A 10 mL sterile syringe was used to carry out this procedure, and the blood obtained was centrifuged and the serum was obtained from the supernatant. Total blood serum glucose, protein, cholesterol, triglycerides, glutamic oxaloacetic transaminase (SGOT) and glutamic pyruvic transaminase (SGPT) were determined using commercial kits. Total plasma follicular stimulating hormones (FSH) and testosterone concentration were measured in this blood serum by radioimmunoassay using two separate commercial kits.

Statistical analysis

All the values of body/organ weight, biochemical estimation and histometrical analysis were expressed in terms of mean ± SD. The different treatment groups were compared with control groups using χ^2-test and Student's *t*-test.[20]

RESULTS

THE RESULTS OF the present study indicate that the orchid has a clear effect on several studied parameters such as:

Effect on body and organ weight

The relative weights of the testes, epididymis, seminal vesicle and ventral prostate were calculated and found to be increased significantly in treated mice when compared with the control group (Table 1). It was also observed that the total bodyweight and vas deferens weight were unchanged in both treated and control mice (Table 1).

Effects on sperm dynamics and density

Table 2 shows that sperm motility (expressed in percentage) and density (sperm count expressed in millions/mL) obtained from cauda epididymis was significantly increased in treated animals when compared with controls. In addition, the percentage comparison range of

Table 1 Effect of *Orchis anatolica* plant treatment on the weights of the body and sex organs of male mice

	Bodyweight (g)	Teste (mg)	Epididymides	Seminal vesicle	Ventral prostate	Vas deferens
Orchis anatolica (n = 20)	37.6 ± 0.31	139 ± 1.57**	50.1 ± 1.36*	67.5 ± 1.69***	37.6 ± 3.18**	13.2 ± 2.8
Control (n = 20)	36.5 ± 0.80	134 ± 0.21	49.3 ± 1.61	58.2 ± 1.38	35.4 ± 4.1	12.8 ± 4.36

Results are expressed as mean ± SD.
*P < 0.05, **P < 0.01, ***P < 0.001. Significant difference from the control group (Student's t-test).

Table 2 Effect of *Orchis anatolica* treatment on sperm dynamics of male mice

	Sperm motility %	Sperm count millions/mL	Sperm viability (%) live : dead
Orchis anatolica (n = 20)	78.26 ± 1.08**	66.7 ± 0.14**	78.13:24.43 ± 0.35**
Control (n = 20)	74.96 ± 1.73	41.56 ± 0.47	71.13:26.43 ± 0.35

Results are expressed as mean ± SD.
**P < 0.001. Significant difference from the control group (Student's t-test).

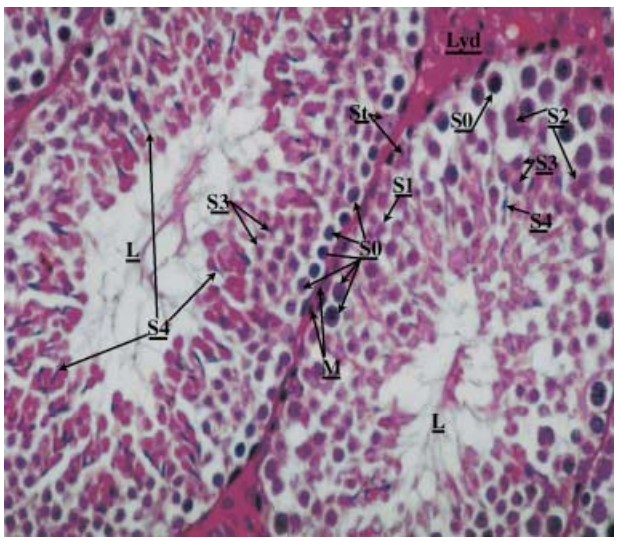

Figure 1 Light microphotograph of normal control mice testicular somniferous tubules showing normal spermatogonial elements. All successive stages of active spermatogenesis are present: spermatogonia (S0), primary spermatocytes (S1) and secondary spermatocytes (S2), spermatids (S3) and spermatozoa (S4). In the center, the tubule lumen (L) and bunches of spermatozoa can be seen adhered to Sertoli cells (St). In the intertubular spaces, healthy Leydig cells (Lyd) and myofibroblasts within the connective tissues (CT) are present. Stained with hematoxylin–eosin (magnification: ×400).

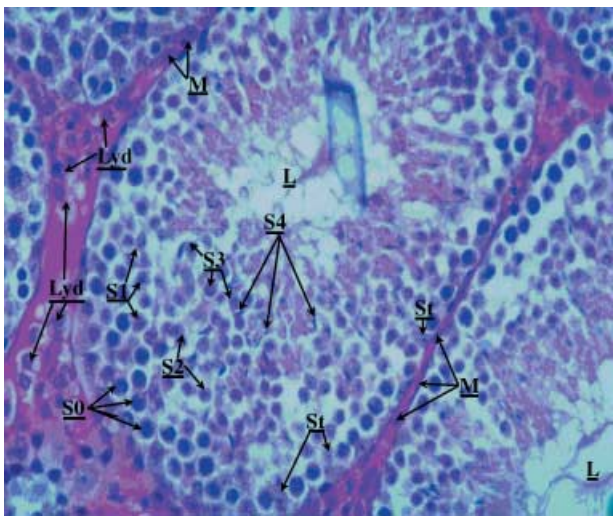

Figure 2 Light microphotograph of testicular somniferous tubules of mice treated with *Orchis anatolica* (5 g/mouse/day) showing active spermatogonial elements. All successive stages of active spermatogenesis are present An increase in spermatogenesis cell density with a increase in germinal epithelium proliferation is observed if compared with Figure 1 (control). The somniferous tubule diameter is increased significantly. An increase in the relative amount of interstitial connective tissue (CT) was observed also with an increase in the density of Leydig cells (Lyd). Stained with hematoxylin–eosin (magnification: ×400).

viable sperms (live to dead ratio) obtained from cauda epididymis was found to be elevated significantly in orchid treated mice in favor of living sperms, indicating higher sperm viability when compared with the control group (Figs 1 and 2).

Effect on testicular cell population

Table 3 shows that in mice receiving orchid root treatment, a significant increase in the density of germinal cell population such as spermatogonia, spermatocytes

Table 3 Effect of *Orchis anatolica* treatment on testicular cell population's dynamics of male mice.

	Germinal cell types				Interstitial cell type			
	Spermatogonia	Spermatocyte (primary)	Spermatocyte (secondary)	Spermatids	Fibroblast	Immature Leydig cell	Mature Leydig cell	Degenerating cell
Orchis anatolica (n = 20)	87.23 ± 4.44*	27.96 ± 2.41**	97.97 ± 3.73***	169.32 ± 6.82***	78.66 ± 1.33**	81.66 ± 1.65**	86.66 ± 0.78***	14.0 ± 0.76**
Control (n = 20)	79.23 ± 0.93	18.85 ± 0.80	64.126 ± 3.51	127.71 ± 4.87	63.83 ± 1.64	65.195 ± 3.47	70.64 ± 1.03	18.34 ± 1.67

Results are expressed as mean ± SD.
*P < 0.05, **P < 0.001 ***P < 0.001. Significant difference from the control group (Student's t-test).

(primary and secondary) as well as spermatids was observed. Similarly, interstitial cell population count including fibroblasts, and mature and immature Leydig cells were also increased to a significant level when compared with controls (Table 3). In contrast, the degenerating cell numbers were reduced in a significant manner, which enhances and validates our results indicated previously (Table 3, Figs 1 and 2).

Effect on biochemical changes analysis

No changes were observed in the serum titer levels of glucose, total cholesterol and triglycerides, as well as in both SGOT and SGPT in both groups (Table 4). However, the plasma levels of both FSH and testosterone were increased to significant levels in the treated group of mice when compared with controls (Table 4).

Effect on male mouse fertility

The number of impregnated female mice fertilized by treated male mice was found to be 95% when compared with 85% of female mice fertilized by male control mice. Table 5 shows that the numbers of both the implantation sites and the viable fetuses were increased significantly when compared with females mated with untreated mice. In addition, the number of resorption sites (dead fetuses found within the dissected uterus) was decreased to 1.5% in females fertilized by treated mice when compared with 5% in female mice fertilized by untreated mice (Table 5). The ratio of male to female newborn mice ratio (number of viable fetuses) was found to be elevated within the female mice group fertilized with treated mice when compared with female fertilized with the control mice group (Table 5).

DISCUSSION

THE *ORCHIDACEAE* FAMILY is the largest plant family to be used in herbal medicine.[2,3] The fact that this genera of plant is made up of diversified chemical constituents interacting in a highly complex manner has led to the use of this plant in herbal medicine, as it contains popular aphrodisiac properties and cannot be reduced to simple and separate ingredients.[9–13]

The male animal model used in the present study has been used previously by several workers to assess the adverse effects of other plant extracts on reproductive functions.[21] In mice, the whole spermatogenic process requires 35 days, out of which spermatozoa require at last 6–7 days of its migration process to reach its final

Table 4 Effect of *Orchis anatolica* treatment on serum biochemistry parameters of male mice

	Glucose mmol	Cholesterol	Triglycerides	Bilirubin	SGOT U/L	SGPT	Testesterone µnmol/L	FSH IU/L
Orchis anatolica (n = 20)	7.74 ± 0.49	0.94 ± 0.06	0.75 ± 0.05	3.7 ± 0.47	33.11 ± 5.5	72.75 ± 4.75	17.83 ± 1.89**	26.7 ± 2.39**
Control (n = 20)	7.58 ± 0.37	0.88 ± 0.1	0.69 ± 0.177	3.5 ± 0.32	27.8 ± 1.43	67.6 ± 4.12	13.92 ± 1.53	21.87 ± 2.55

Results are expressed as mean ± SD.
FSH, follicle stimulating hormone; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase.
*P < 0.05, **P < 0.001. Significant difference from the control group (Student's t-test).

Table 5 Effect of *Orchis anatolica* treatment on fertility outcome in control female mice

	No. male	No. female	No. pregnant females (%)	No. implantation sites	No. viable fetusesIn uetrus	No. resorption/ total no. implantation	No. viable fetuses male/female ratio at birth
Orchis anatolica (n = 10)	10	20	19/20 (95%)	11.37 ± 1.31**	10.83 ± 1.54***	3/206 (1.45%)	
Orchis anatolica (n = 10) after birth	10	20	19/20 (95%)				122:75 (61.9:38.1)**
Control (n = 10)	10	20	17/20 (85%)	9.62 ± 1.66	8.28 ± 1.16	7/140 (5%)**	
Control (n = 10) after birth	10	20	17/20 (85%)				66:70 (48.5:51.5)

Results are expressed as mean ± SD.
**P < 0.001, ***P < 0.001. Significant difference from the control group (Student's t-test).

transitional stage within the epididymides.[22] The *Orchis anatolica* roots were chosen for the present study as this plant has not been explored sufficiently by researchers using animal models.

The present investigation showed that *Orchis anatolica* given orally to mice promoted an increase in the diversified fertility parameters of the male reproductive system through an unexplained mechanism. This was partially shown by the findings listed in Table 1, indicating that male mice receiving this treatment showed a significant increase in most of reproductive system organ weights mentioned. It has been postulated, however, that reproductive organ weight and function such as testes, epididymes and seminal vesicles, are closely regulated by androgens.[23] This might indicate that this plant could act directly or indirectly on the pituitary gland, the main androgenic gland regulator, leading to an increase in the hormone that controls and regulate spermatogenesis. This could enforce the proposed hypothesis mentioned earlier 'that orchids could enhance the reproductive system functional ability' and, more practically, increasing sperm formation in orchid treated male mice as shown by our findings.

Furthermore, it is well known that the process of spermatogenesis and the function of accessory reproductive organs are androgen dependent. Therefore, an increase in androgen production would reflect the increase in number of mature Leydig cells and their functional status shown through our experiment. In the present study, the number of degenerated Leydig cells was significantly decreased, enforced by the increase of androgen levesl observed in the present study. This is further confirmed and reflected by an increase in the number of spermatocytes (primary and secondary) and spermatids as they are completely androgen dependent at this stage.[24] The weight increase of the reproductive organs observed in treated animals further confirmed this rise in the androgen levels, leading to a vicious circle mechanism. In addition, the increase in the sperm motility obtained from cauda epididymis as observed in the treated mice group could lead to a hypothesis that oxidative phosphorylation enzymes might participate in this spermatogenic process, which might enhance the effects of the orchid.

The results of the present study also showed that ingestion of the orchid extract by adult male mice increases the number of impregnations in the normal untreated control female mice when left to mate. This is indicated in Table 5, which shows an increase in the number of implantation sites and the number of viable fetuses born by impregnated females. The only explanation for this fact is that this increase might be a result of the fact that sperm motility and sperm density, as well as viability, were significantly increased, as indicated in our results.

We can hypothetically conclude that the *Orchis anatolica* diet might contain a compound within its structure that might influence the male reproductive system. This could directly or indirectly lead to an increase in the potential of fertility in male mice, mainly by acting directly on the pituitary gland and influencing its secretion and/or the pituitary gland–spermatoginic axis. Further studies are required and arein progress to isolate and identify the active principle(s) that acts upon this axis, influencing and increasing this process and the precise mode of its action. Further study might be required to evaluate the treatment effect of *Orchis anatolica* at the intracellular as well as the DNA level.

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